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A bioassay for brassinosteroid activity based on the *in vitro* fluorimetric detection of nitric oxide production

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ABSTRACT

Recent studies have shown that low concentrations of brassinolide induce a rapid generation of nitric oxide in mesophyll cells of maize leaves, which can be easily detected by fluorimetric methods. In this work we describe a series of natural and synthetic brassinosteroids that are able to trigger *in vitro* NO production in tomato cells that exhibits dose–response behavior. We propose that this effect can be used to develop a new rapid and very sensitive bioassay for brassinosteroid activity that offers several advantages when compared to the current methodologies.

1. Introduction

The role of steroids as hormones in mammals is known since the 1930s, later their endocrine function in insects and fungi was well stablished. Although plants are able to produce a wide range of steroids, it was only in 1979 when researchers from the United States Department of Agriculture described the structure of brassinolide (**1**), a steroidal lactone isolated from pollen of rapeseed (*Brassica napus*), with a remarkable activity on plant growth [1]. Since then, a large number of structurally related steroids, collectively named as brassinosteroids (BRs), were found in all plant species: higher plants, ferns and algae [2]. BRs proved to have regulatory activity on the growth and development of plants [3,4], such as stimulation of cell division, vegetative growth, reproduction and tolerance to stress and pathogens [5–7]. Due to the low concentration of BRs in plant tissues, their chemical synthesis and that of a wide variety of analogs was mandatory in order to obtain the compounds needed to undertake studies on BRs biosynthesis, metabolism, the underlying molecular mechanisms involved in signal transduction [8–10] and the subsequent genomic responses [11,12]; nowadays, these processes are among the best studied in plant biology.

Once the presence of BRs in plants had been established, the suitability of these new substances for improving the yield of economically important crops was explored. There are many reports, which have been summarized in monographs [13–15], about the beneficial effects of exogenously applied BRs on yield, general vigor, and stress tolerance of commercially important crops [16,5].

Among the major constraints for large-scale use of BRs in crop production are their high cost and limited half-life in the environment. However, progress in the chemical synthesis of long-lasting analogues and non-steroidal mimetics [17] has led to economically feasible approaches that have brought their practical application in agriculture within reach. A key application of BRs that is currently under development is their use as growth factors in techniques involving plant propagation from cuttings, grafting, micropropagation and tissue culture, especially of valuable recalcitrant species [18–20].

In the search for new synthetic BRs with potential use in agriculture, it is vital that hormonal activity is evaluated with a reliable bioassay. Several bioassays have been developed [21], but the most prevalent are the bean second internode elongation assay (BSIE), and the rice leaf lamina inclination test (RLIT).

The bean internode elongation assay uses bean (*Phaseolus vulgaris*) seedlings when the second internode from the root-shoot junction is still growing [22]. BRs characteristically provoke elongation in a dose–response manner, accompanied by curvature, swelling and splitting. The rice leaf lamina inclination test, which is used extensively for the identification of BRs during purification protocols and for determining the potency of natural and synthetic

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BRs, is based on the bending of the lamina of rice leaves away from the sheath when exposed to BRs [23]. Explants consisting of lamina, lamina joint, and leaf sheath from etiolated rice seedlings are floated on test solutions and the bend of the leaf lamina is measured. In a modified protocol, intact rice (*Oryza saliva*) seedlings are used and the test solutions are applied as a microdrop at the junction between the lamina and the sheath [24]. The bending seems to involve the activation of genes that modulate cell division at the lamina joint [25].

These bioassays are quite simple and inexpensive, but their lengthiness (two to four days are needed, not including the one week span required to grow the seedlings) renders them inappropriate for high throughput screening of large collections of BRs.

Recently, it has been shown that low concentrations of brassinolide induce a rapid generation of nitric oxide in mesophyll cells of maize leaves, which can be easily detected by fluorimetric methods [26]. Guided by these results, here we present a preliminary study showing that BRs-induced NO production in cultured plant cells can be developed into a new rapid and sensitive bioassay for measuring brassinosteroid activity.

2. Experimental

2.1. Tested compounds

Brassinolide (**1**) was purchased from Sigma–Aldrich Co. Other natural brassinosteroids and analogs tested in this work were synthesized as previously described [27–29].

2.2. Rice lamina inclination test (RLIT) [25]

Rice seedlings (*Oryza sativa*, cv Chui) were washed with ethanol (1 min) and water and then left in water at 30 °C for two days (with a 16 h photoperiod). Germinated seeds were cultivated in agar under the same growing conditions for four days. Twenty intact seedlings (4–5 cm tall) were inoculated with 0.5 μL of the test compound solution (in ethanol) just under the second leaf joint. Seedlings were kept in the dark (at 30 °C), and after 48 h the magnitude of the induced angle between the leaf and the sheath was measured. Activity for each compound at a given dose is reported as:

(Mean angle of treated plant
– mean angle of control)/mean angle of control

where ethanol was injected as the control.

2.3. BRs-induced nitric oxide production

Tomato (*Solanum lycopersicum* 'Money Maker'; line Msk8) suspension-cultured cells were grown at 24 °C in the dark at 125 rpm in Murashige and Skoog medium (Duchefa), supplemented with 5.4 mM naphthylacetic acid, 1 mM 6-benzyladenine, and vitamins (Duchefa). After 5 days of culture cells were transferred to a multiwell plate and pretreated for 30 min with the NO-specific fluorophore 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM-DA at 0,5 μM, and subsequently incubated in the presence or absence of the different BRs (previously dissolved in DMSO) in a final volume of 100 μL.

Fluorescence was measured with a Fluoroskan Acsent fluorometer (Thermo Electron Company, Finland), using D480-40 and D525-30 filters for excitation and emission, respectively, every 2 min for 2 h in each well. Cell suspensions were kept at 25 °C and 120 rpm during measurements, which were made in triplicate. Activity for each compound at a given dose is defined as:

Slope of the fluorescence vs. time curve/slope of
the fluorescence vs. time curve of the control.

where DMSO was injected as the control.

2.4. Synthesis

2.4.1. General

All the reagents were purchased from Sigma–Aldrich Chemical Co. Melting points were determined on a Fisher Johns apparatus and are uncorrected. All NMR spectra were recorded on a Bruker AM-500 (500 MHz for ^{1}H, 125.1 MHz for ^{13}C and 470.5 MHz for ^{19}F). Chemical shifts (Δ) are given in ppm downfield from TMS as the internal standard for ^{1}H and ^{13}C, and downfield from CCl_3F for ^{19}F). Coupling constant (J) values are in Hz. For unambiguous assignment of both ^{1}H and ^{13}C signals gHSQC and gHMBC spectra were measured using standard parameters sets and pulse programs. All solvents and reagents were of analytical grade. ESI-HRMS were measured on a Bruker micrOTOF-Q II. Analysis of all new compounds yielded satisfactory combustion data (purity $\geqslant$98%) on an Exeter CE 440 Elemental Analyzer. Optical rotations were measured on a Jasco J-815 spectropolarimeter. IR spectra were recorded on a Nicolet iS50 FT-IR spectrometer and are shown as Supplementary Data.

2.4.2. Synthesis of (22E)-2β-fluoro-5α-stigmasta-22-en-3,6-dione (*15*)

200 mg (0.43 mmol) of (22*E*)-3-acetoxy-5α-stigmasta-2,22-dien-6-one **14** (obtained from stigmasterol, as previously described [28]) were dissolved in 10 mL of dry dichloromethane in a polypropylene round bottom flask under an argon atmosphere. 108 mg (0.56 mmol) of xenon difluoride were added. The reaction was stirred for 3 h at room temperature and poured into ice. The mixture was extracted with dichloromethane (3 × 15 mL) and the organic layer was washed with brine, water, dried with sodium sulfate and evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel (hexane/EtOAc 95:5), yielding 34% (65 mg, 0.15 mmol) of (22*E*)-2α-fluoro-5α-stigmasta-22-en-3,6-dione [28]. Further elution gave 97 mg (51% yield) of the desired (22*E*)-2β-fluoro-5α-stigmasta-22-en-3,6-dione **15** M.p.: 114–115 °C. $[\alpha]_D^{20}$−44 (c 0.25, $CHCl_3$). ^{1}H-NMR ($CDCl_3$): 0.71 (H-18, 3H, *s*); 0.79 (H-26, 3H, *d*, J = 6.8); 0.80 (H-29, 3H, *t*, J = 7.0); 0.84 (H-27, 3H, *d*, J = 5.9); 1.02 (H-21, 3H, *d*, J = 8.6); 1,03 (H-19, 3H, *s*); 2,70 (H-5α, 1H, *dd*, J = 3.9 and 12.9); 3.03 (H-4β, 1H, *dm*, J = 13.3); 4.75 (H-2α, 1H, *dt*, J = 4,5 y $^{2}J_{HF}$ = 49.0); 5.03 (H-23, 1H, *dd*, J = 8.5 y 15.3); 5.14 (H-22, 1H, *dd*, J = 8.5 y 15.3). ^{13}C-NMR ($CDCl_3$): 12.2 and 12.2 (C-18 and C-29); 14.4 (C-19, *d*, $^{4}J_{CF}$ = 4.6); 18.9 (C-26); 21.0 and 21,1 (C-21 and C-27); 21.6 (C-11); 24.0 (C-15); 25.3 (C-28); 28.6 (C-16); 31.8 (C-25); 34.0 (C-4); 37.4 (C-8); 39.2 (C-12); 40.3 (C-20); 41.0 (C-10); 42.8 (C-13); 44.0 (C-1, *d*, $^{2}J_{CF}$ = 20.2); 46.2 (C-7); 51.2 (C-24); 54.0 (C-9); 55.8 (C-17); 56.5 (C-14); 56.9 (C-5); 91.3 (C-2, *d*, $^{1}J_{CF}$ = 180.8); 129.7 (C-23); 137.8 (C-22); 206.0 (C-3, *d*, $^{2}J_{CF}$ = 18.7); 207.7 (C-6). ^{19}F-NMR ($CDCl_3$): −182.0 (*dddd*, $^{2}J_{HF}$ = 49.2; $^{3}J_{HF}$ = 37.1 y 19.5; $^{4}J_{HF}$ = 1.7). MS (EI): *m*/*z* (rel.int.): 444 $[M]^+$ (10); 401 (15); 331 (16); 303 (29); 55 (100).

2.4.3. Synthesis of (22E)-2β-fluoro-3α-hydroxy-5α-stigmast-22-en-6-one (*16*)

The fluorodiketone **15** (105 mg, 0.21 mmol) was dissolved in 5 ml of dry CH_2Cl_2 under an argon atmosphere and 15 mg of tetrabutylammonium borohydride was added in one portion. The reaction was stirred for 1 h and the organic layer was washed successively with NH_4Cl (s.s.), brine, and water, and then dried over Na_2SO_4. The solvent was evaporated and the resulting products were separated by silica gel column chromatography (hexane/AcOEt gradient) to yield 90% (95 mg, 0.21 mmol) of